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Rapid report

Tobacco BY-2 cell-free extracts induce the recovery of microtubule nucleating activity of inactivated mammalian centrosomes

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Abstract

The structure and the molecular composition of the microtubule-organizing centers in acentriolar higher plant cells remain unknown. We developed an in vitro complementation assay where tobacco BY-2 extracts can restore the microtubule-nucleating activity of urea-inactivated mammalian centrosomes. Our results provide first evidence that soluble microtubule-nucleating factors are present in the plant cytosolic fraction. The implication for microtubule nucleation in higher plants is discussed.

Keywords: Microtubule; Nucleation; Microtubule-organizing center; Centrosome; Higher plants

In eukaryotes, the assembly and the distribution of microtubules mainly result from the activity of microtubule-organizing centers (MTOCs). In many cells MTOCs appear as discrete organelles from which microtubules radiate, such as centrosomes in mammalian cells. Higher plant cells do not possess structurally defined MTOCs [1]. In these cells different microtubule populations are organized in specific patterns throughout the cell cycle; specifically, nuclear-associated microtubules, cortical microtubules, preprophase band microtubules, spindle microtubules and the phragmoplast microtubules [2]. In all higher plant cells examined so far, none of the microtubule arrays emanate from a centriolar-like structure. Indeed neither the location nor the mechanisms of plant microtubule nucleation are known. The fact that isolated plant nuclei can promote microtubule assembly [3] strongly suggests that the nuclear surface possesses microtubule nucleation sites in higher plant cells. So far the nuclear surface is the only plant MTOC that has been functionally characterized. The presence of γ-tubulin, a universal MTOC marker, on the plant nuclear surface reinforces this hypothesis. However, the striking association of γ-tubulin with all microtubule arrays remains unexplained [4,5]. This may suggest the presence of dispersed microtubule nucleation sites in the plant cytoplasm, besides an aggregated form at specific MTOC-like domains such as the nuclear surface. Such a situation is unique to plant cells and raises

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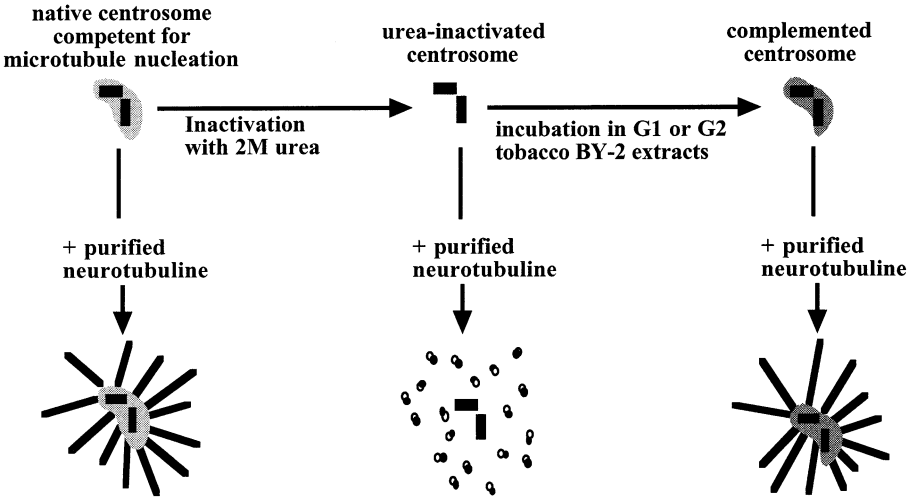

Fig. 1. Procedure of the complementation assay. During the complementation assay, urea-inactivated centrosomes are incubated with G_1 or G_2 tobacco BY-2 extracts. Soluble factors present in these extracts are recruited by the centrosomes, which are then called complemented centrosomes. The ability of both complemented centrosomes and urea-inactivated centrosomes to nucleate microtubules is tested in the presence of purified neurotubulin, and compared with the microtubule nucleation capacity of native centrosomes.

the question of what is the nature of the MTOC in acentriolar higher plant cells.

It has been proposed that part of the mechanisms for microtubule nucleation are common to plant and animal cells. To investigate this issue we took advantage of the centrosome complementation assay previously designed [6] in which the nucleating activity of urea-inactivated centrosomes can be restored by the recruitment of soluble components present in *Xenopus* extracts. We used this assay to ask the question whether higher plant cells also contain factors that are able to reconstitute the microtubule nucleating ability of mammalian inactivated centrosomes. The procedure we used is summarized in Fig. 1. Centrosomes (10^8/ml) were purified from human KE37 lymphoid cells. Centrosomes were diluted in BRB80 (80 mM Pipes (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA) containing 2 M urea and incubated for 1 h at 4°C. Urea-treated centrosomes were dialyzed against BRB80 for 1 h at 4°C, resuspended in BRB80 containing 40% (v/w) sucrose and stored as aliquots (approximately 10^7/ml) at −80°C. It has been previously reported that such urea-treated centrosomes, also called inactivated centrosomes, are no more able to nucleate microtubules [6,7]. To test the efficiency of the urea treatment on the centrosomes, urea-treated centrosomes (1 μl) were mixed with purified neurotubulin (20 μM) diluted in cold BRB80 [8] containing 1 mM GTP and 10^{-6} M oryzalin. The final volume was adjusted to 50 μl with BRB80. The mixture was incubated for 15 min at 37°C. Reactions were stopped by dilution in 1 ml of prewarmed (30°C) 0.25% (v/v) glutaraldehyde, 1 mM GTP in BRB80, and processed for immunocytochemistry [8]. As shown in Fig. 2A, native centrosomes nucleated numerous microtubules in the experimental conditions we used. In contrast, urea-treated centrosomes lost their capacity to nucleate microtubule when assayed in the same conditions (Fig. 2B). In very few cases (0,04%), 2–5 microtubules emanated from these centrosomes. No free autoassembled microtubules were detected. These inactivated centrosomes were used in complementation assays with tobacco extracts.

Plant extracts were prepared from miniprotoplasts of synchronizable tobacco BY-2 cells (Nicotiana tabacum cv Bright Yellow-2). Tobacco BY-2 cells were cultured in suspension and synchronized as described [9]. Seven-day-old cells were used as a source of cells in G_1 phase. At this time cell suspension was in a stationary growth phase and the mitotic index was close to 0%. Cells in G_2 were obtained after synchronization with aphidicolin, a inhibitor of DNA polymerase α. Cells were collected 2 h after drug removal, when 90–92% of the cells were in G_2. Progression of cells through the cell cycle was followed by DNA staining with Hoechst dye 33258 (0.1 μg/ml in 0.2%

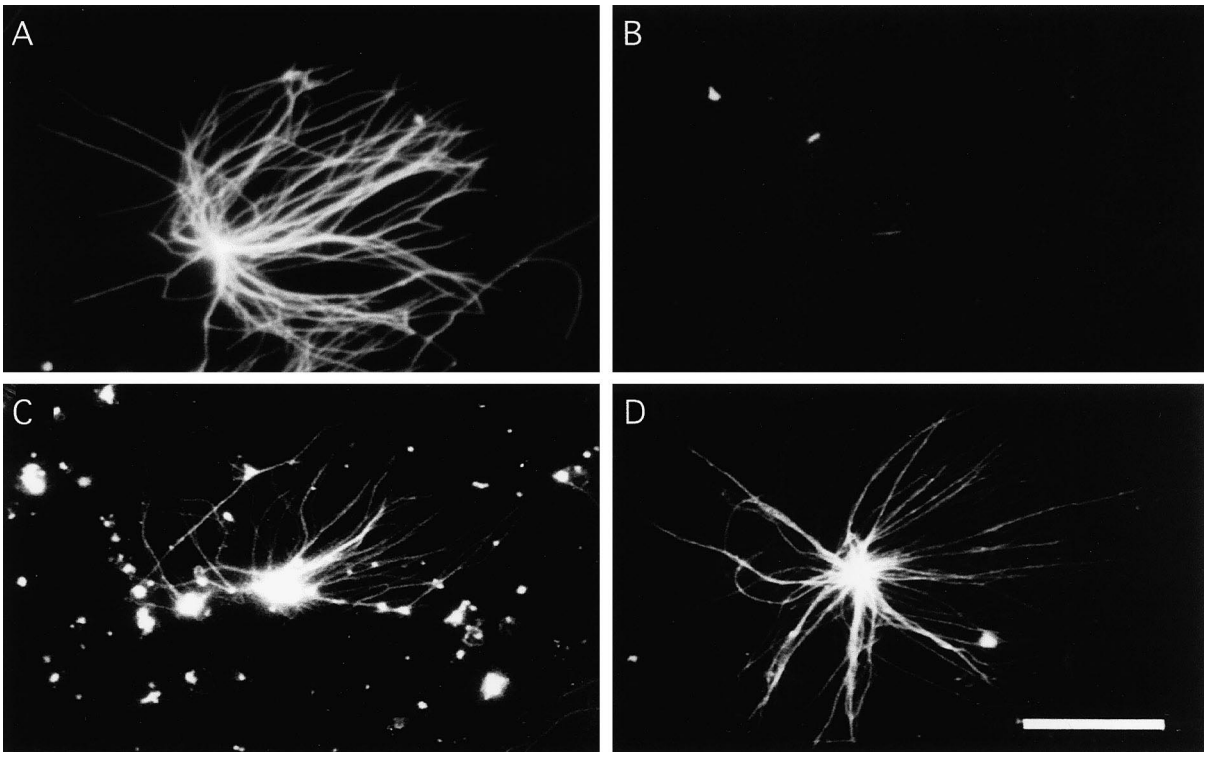

Fig. 2. Microtubule nucleating activity of native and inactivated centrosomes and centrosomes complemented in tobacco BY-2 extracts, analyzed by immunofluorescence using tubulin antibodies. Native centrosomes nucleate microtubules (A), whereas urea-inactivated centrosomes are no more able to nucleate microtubules (B). Inactivated centrosomes complemented in G_1 (C) or G_2 (D) tobacco BY-2 extracts recovered their ability to promote microtubule assembly in pure tubulin solution (20 μM). Bar = 10 μm.

(v/v) Triton X-100). Interphase extracts were prepared from tobacco cells both in G_1 and in G_2. Cells were collected by centrifugation ($100 \times g$, 5 min) and incubated for 5 h at 30°C in the dark under agitation (130 rpm) in buffer A (25 mM Mes (pH 5.5), 0.45 M mannitol, 8 mM $CaCl_2$, 0.15% (w/v) pectolyase Y-23, 0.2% (w/v) macerozyme R-10, 3% (w/v) cellulase Onozuka RS). Protoplasts were centrifuged ($100 \times g$, 5 min) and washed three times at 10°C in buffer B (25 mM Mes (pH 5.5), 17% (w/v) sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA). Miniprotoplasts were prepared according to Sonobe [10]. Protoplasts were evacuolated by centrifugation ($11\,000 \times g$, 90 min, 10°C) in buffer C (25 mM Pipes (pH 5.8), 10 mM $MgCl_2$, 0.6 M mannitol, 30% (v/v) Percoll). All further steps were performed at 4°C. The supernatant was removed and the pellet of miniprotoplasts was washed twice in buffer D (25 mM Pipes (pH 5.8), 0.6 M mannitol) and once in BRB80Ac (80 mM Pipes (pH 6.94), 33 mM K-acetate, 5 mM $MgCl_2$, 10 mM EGTA, 0.5 mM EDTA). The cell cycle stage of miniprotoplasts was determined by DNA staining. Miniprotoplasts were suspended in a minimal volume of BRB80Ac containing 0.5 mM GTP and protease inhibitors (10 μM leupeptin, 10 μM pepstatin, 1 mM DTT, 1 mM PMSF, 1 mM aprotinin). They were lysed by forcing through 22-gauge and 26-gauge needles several times. The lysate was clarified by centrifugation ($100\,000 \times g$, 60 min, 4°C). An ATP-regenerating system (10 mM creatine phosphate, 80/μg.m creatine phosphokinase, 1 mM ATP) [11] was added to the supernatant. Samples were stored as aliquots in liquid nitrogen. Protein extracts from miniprotoplasts in G_1 stage (G_1 extracts) and in G_2 stage (G_2 extracts) had a concentration of 30–35 mg/ml and 40–45 mg/ml, respectively. Urea-treated centrosomes (1 μl) were incubated at 30°C for 60 min in either G_1 or G_2 extracts (600 μg) containing 1 mM ATP. One-μl aliquots of the samples were used for microtubule nucleation assays in the same conditions as described above.

As shown in Fig. 2C,D, urea-inactivated centrosomes recovered their ability to nucleate microtubules following preincubation in interphase tobacco BY-2 extracts. We called these centrosomes complemented centrosomes. Preincubation of inactivated centrosomes in tobacco extract was made in the presence of 10^{-6} M oryzalin. At this concentration oryzalin specifically depolymerizes plant microtubules and inhibits plant tubulin assembly, but it has no effect on brain microtubule assembly [12]. These results demonstrate that the complementation of the inactivated centrosomes strictly depended on the presence of soluble plant factors, and did not require the presence of endogenous plant microtubules. We estimated that 13.75% and 15.48% of urea-inactivated centrosomes recovered their capacity to nucleate microtubules after incubation with G_1 and G_2 tobacco BY-2 extracts, respectively. Microtubules nucleated by G_1 and G_2 extracts had a mean length of 16.1 μm and 19.6 μm, respectively. Although our experimental conditions did not allow the precise quantification of microtubules nucleated by the complemented centrosomes, we noticed that G_2-complement centrosomes usually had a higher density of nucleated microtubules compared with G_1-complemented centrosomes. Recovery of the microtubule nucleating capacity of centrosomes was dependent on the amount of tobacco BY-2 extract added to the inactivated centrosomes. We found that a minimum of 600 μg of tobacco BY-2 extract protein was required to restore the microtubule nucleating capacity of 10^5 inactivated centrosomes. In all of the experiments we performed, the nucleating activity of complemented centrosomes appeared to be lower than that of native centrosomes (compare Fig. 2A,D).

Herein we demonstrated that tobacco BY-2 extracts were able to restore the microtubule nucleating capacity of urea-treated centrosomes. These results suggest that soluble factors present in tobacco BY-2 extracts are involved in microtubule nucleation. The mechanism by which these soluble microtubule nucleating factors are able to complement mammalian centrosomes is not known. Interestingly, the complementation of inactivated centrosomes by either tobacco BY-2 extracts or by *Xenopus* egg extracts [6] shares two common features. In both experiments, (i) the recruitment of microtubule nucleating factors by inactivated centrosomes occurred in the absence of microtubules and (ii) a minimal amount of protein extract was required for the recovery of microtubule nucleation by centrosomes. Whether these factors simply bind to centrosomes or whether they are specifically recruited by centrosomes remains to be determined. It is noteworthy that in our study higher plant proteins were able to restore the microtubule nucleation function of mammalian centrosomes. Given that several antibodies raised against mammalian centrosomes cross-react with plant polypeptides [3,4,13], it has been suggested that some of the proteins involved in microtubule nucleation may be conserved between animal and plant kingdoms in spite of major differences in the structure of the MTOCs. Our data provide functional evidence that reinforce this hypothesis.

The identification of the tobacco BY-2 proteins that bind to the centrosomes is of crucial importance for our understanding of microtubule nucleation in higher plant cells. Unfortunately, the small amount of tobacco BY-2 proteins recruited by inactivated centrosomes from the extracts has made biochemical analysis difficult. Tobacco BY-2 extracts used in complementation assays probably contain MAPs. We previously demonstrated that tobacco BY-2 protein fractions enriched in putative MAPs affect the microtubule nucleation from mammalian centrosomes at MAP-fraction/tubulin ratios (w/w) of 1:10 to 1:3 [8]. Given the fact that MAPs represent 0.4% of plant soluble proteins [14], it seems unlikely that MAPs are responsible for the microtubule nucleation of complemented centrosomes. Indeed it has been shown that purified neural MAPs can not complement urea-inactivated centrosomes [6]. Although we cannot exclude the possibility that MAPs participate in the complementation process, we believe that other uncharacterized tobacco BY-2 proteins are necessary to restore the microtubule nucleating ability of inactivated centrosomes in complementation assays. It would be of interest to determine whether γ-tubulin is recruited by inactivated centrosomes. γ-Tubulin is a unique tubulin family that does not assemble into microtubules [15]. Several lines of evidence strongly suggest that γ-tubulin is a universal component of the MTOC and that it is essential for microtubule nucleation [16–19]. γ-Tubulin is found in the cytoplasm of both *Xenopus* eggs and somatic mam-

malian cells as part of large soluble multiprotein complexes called γ-somes [20–22]. Very recently it has been shown that in vitro the recruitment of both γ-tubulin complex and as yet unidentified factors is sufficient to restore the microtubule nucleating activity of inactivated centrosomes [23]. In higher plants the function of γ-tubulin in microtubule nucleation is still unclear. We know from immunoblot analysis that γ-tubulin is present in the tobacco BY-2 extracts we used in our assays (data not shown). However, we could not clearly demonstrate whether γ-tubulin is recruited by the urea-inactivated centrosomes. More experiments using purified γ-tubulin or purified γ-tubulin complexes are required to answer this question. In this context, the use of the complementation assay should provide insights into the functions of γ-tubulin and as yet unidentified factors in plant microtubule nucleation.

An important question that remains to be answered is whether the plant microtubule nucleating material is competent for microtubule assembly in its soluble form in vivo. Considering the complex organization of microtubule arrays in higher plant cells, we imagine that the soluble microtubule nucleating factors may have two distinct and not exclusive functions in vivo. First they could be recruited by a preexisting MTOC, and consequently increase its microtubule nucleation capacity. For example, this process could participate in the increase microtubule nucleation capacity of the plant nuclear surface in G_2. Second, they could be redistributed in specific compartments within the cell and thus generate new microtubule nucleation sites, such as the spindle poles in mitosis. In this context it is possible that the soluble pool of microtubule nucleating material, by alternating between an aggregated form associated with the nuclear envelope and the plasma membrane, and a dispersed form in the cytosol, is important for the regulation of microtubule nucleation in higher plant cells. It has been suggested that the recruitment of microtubule nucleating components is one of the pathways by which the nucleating activity of centrosomes is regulated in mammalian cells [20,21,24,25]. The functions of the soluble form of the plant microtubule nucleation material in vivo and whether its redistribution is involved in the regulation of microtubule nucleation in higher plant cells are important issues that have to be examined more carefully in future work.

References

[1] J. Marc, Microtubule organizing centers in plants, Trends Plant Sci. 2 (1997) 223–230.

[2] A.M. Lambert, C.W. Lloyd, The higher plant microtubule cycle, in: J.S. Hyams, C.W. Llyod (Eds.), Microtubules, Wiley Liss, New York, pp. 325–341.

[3] V. Stoppin, M. Vantard, A.C. Schmit, A.M. Lambert, Isolated plant nuclei nucleate microtubule assembly: the nuclear surface of higher plants has centrosome-like activity, Plant Cell 6 (1994) 1099–1106.

[4] B. Liu, J. Marc, H.C. Joshi, B.A. Palevitz, A γ-tubulin related protein associated with the microtubule arrays of higher plants in a cell-cycle dependent manner, J. Cell Sci. 104 (1993) 1217–1228.

[5] H.C. Joshi, B.A. Palevitz, γ-tubulin and microtubule organization in plants, Trends Cell Biol. 6 (1996) 41–43.

[6] B. Buendia, G. Draetta, E. Karsenti, Regulation of the microtubule nucleating activity of centrosomes in Xenopus egg extracts: role of cyclin A-associated protein kinase, J. Cell Biol. 116 (1992) 1431–1442.

[7] C. Klotz, M.C. Dabauvalle, M. Paintrand, T. Weber, M. Bornens, E. Karsenti, Parthenogenesis in Xenopus eggs requires centrosomal integrity, J. Cell Biol. 110 (1990) 405–415.

[8] V. Stoppin, A.M. Lambert, M. Vantard, Plant microtubule-associated proteins (MAPs) affect microtubule nucleation and growth at plant nuclei and mammalian centrosomes, Eur. J. Cell Biol. 69 (1996) 11–23.

[9] T. Nagata, K. Okada, I. Takebe, Mitotic protoplasts and their infection with tobacco mosaic RNA encapsulated in liposomes, Plant Cell Rep. 1 (1982) 250–252.

[10] S. Sonobe, Cytochalasin B enhances cytokinetic cleavage in miniprotoplasts isolated from cultured tobacco cells, Protoplasma 155 (1990) 239–242.

[11] M.A. Felix, J. Pines, T. Hunt, E. Karsenti, A post-ribosomal supernatant from activated Xenopus eggs that displays post-transcriptionally regulated oscillation of its cdc2+ mitotic kinase activity, EMBO J. 8 (1989) 3059–3069.

[12] L.C. Morejohn, T.E. Bureau, J. Mole-Bajer, A.S. Bajer, D.E. Fosket, Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro, Planta 172 (1987) 252–264.

[13] V. Chevrier, S. Komesli, A.C. Schmit, M. Vantard, A.M. Lambert, D. Job, A monoclonal antibody raised against mammalian centrosomes and screened by recognition of plant microtubule-organizing centers identifies a pericentriolar component of different cell types, J. Cell Sci. 101 (1992) 823–835.

[14] M. Vantard, P. Schellenbaum, A. Fellous, A.M. Lambert,

Characterization of maize microtubule-associated proteins, one of which is immunologically related to tau, Biochemistry 30 (1991) 9334–9340.

[15] C.E. Oakley, B.R. Oakley, Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*, Nature 338 (1989) 662–664.

[16] B.R. Oakley, C.E. Oakley, Y. Yoon, M.K. Jung, Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*, Cell 61 (1990) 1289–1301.

[17] T. Stearns, L. Evans, M. Kirschner, Gamma-tubulin is a highly conserved component of the centrosome, Cell 65 (1991) 825–836.

[18] Y. Zheng, M.K. Jung, B.R. Oakley, Gamma-tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with centrosome, Cell 65 (1991) 817–823.

[19] H.C. Joshi, M.J. Palacios, L. McNamara, D.W. Cleveland, γ-tubulin is a centrosomal protein required for cell-cycle dependent microtubule nucleation, Nature 356 (1992) 80–83.

[20] M.A. Felix, C. Antony, M. Wright, B. Maro, Centrosome assembly in vitro: role of γ-tubulin recruitment in Xenopus sperm aster formation, J. Cell Biol. 124 (1994) 19–31.

[21] T. Stearns, M. Kirschner, In vitro reconstitution of centrosome assembly and function: the central role of γ-tubulin, Cell 76 (1994) 623–637.

[22] Y. Zheng, M.L. Wong, B.M. Alberts, T. Mitchison, A γ-tubulin ring complex purified from the unfertilized egg of *Xenopus laevis* can nucleate microtubule assembly in vitro, Nature 378 (1995) 578–583.

[23] M. Moritz, Y. Zheng, B.M. Alberts, K. Oegema, Recruitment of the γ-tubulin ring complex to Drosophila salt-stripped centrosome scaffolds, J. Cell Biol. 142 (1998) 775–786.

[24] K. Ohta, N. Shiina, E. Okumura, S. Hisanaga, T. Kishimoto, S. Endo, Y. Gotoh, E. Nishida, H. Sakai, Microtubule nucleating activity of centrosomes in cell-free extracts from Xenopus eggs: involvement of phosphorylation and accumulation of pericentriolar material, J. Cell Sci. 104 (1993) 125–137.

[25] S.J. Doxsey, P. Stein, L. Evans, P.D. Calarco, M. Kirschner, Pericentrin, a highly conserved centrosome protein involved in microtubule organisation, Cell 76 (1994) 639–650.